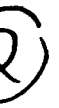

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Trimester Report 3/15/91-7/12/91

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I. Work Summary

Over the last several months we have especially focused on two projects in the laboratory related to discoveries we made early in the spring relating to: 1. the interactions that LPS undergoes before binding to anti-LPS immunoglobulins in hyperimmune antisera to rough mutant E. coli J5, and 2. IgG titers to the O-polysaccharide of LPS in the screened plasma of normal blood donors. Both of these projects represent offshoots from our overall aim of purifying the substances in inflammatory serum which bind LPS.

This month represents the termination of our second year of our grant. We are now in full stride and I we think are working very efficiently. In addition to this brief trimester report I will submit an annual report within the next several months containing a summary of the last year's work together with our publications.



A. Purification of LPS binding factors in inflammatory serum

As noted above we concentrated this trimester on the changes that LPS undergoes in normal serum and how this altered LPS interacts with immunoglobulin. We did, however, achieve several of our goals for the trimester relating to the purification of LPS binding factors in inflammatory serum.

We completed the laborious (and unpleasant) task of radiolabeling with tritium a panel of gram negative bacteria consisting of the 10 different strains of E. coli which most commonly cause infections. Each strain was grown in 100 mls broth containing 10-25 millicuries of $^{3}$H-acetate to radiolabel the bacteria. The radiolabeled LPS component of each bacterial strain was then extracted by the hot phenol method, treated with enzymes to remove trace contaminating nucleic acids and proteins, ultracentrifuged, lyophilized, and then standardized by weight and limulus lysate activity. This procedure in our hands yields LPS with specific activities of 5,000-20,000 CPM/ug. Higher specific activities can be obtained by labeling the LPS exogenously with other isotopes, but these techniques result in an altered LPS molecule which potentially changes the binding characteristics. We therefore prefer to endogenously radiolabel the LPS. We now have a panel of LPS from clinically important gram negative bacterial strains which should be good for the next several years. The specific activity of the LPS in our panel is shown on the top of the next page.



| E. coli strain | Specific activity (CPM/ug LPS) |
|---|---|
| O1 | 7,140 |
| O2 | 13,800 |
| O4 | 10,480 |
| O6 | 7,200 |
| O7 | 12,150 |
| O8 | 10,980 |
| O16 | 17,100 |
| O18 | 22,433 |
| O25 | 11,040 |
| O75 | 4,700 |
| O111 | 15,800 (labeled with $^{3}$H-galactose several months earlier) |

We also developed a Western blotting assay to measure the binding of LPS to proteins which had been previously blotted onto nitrocellulose. The assay is performed by running the protein solution on an SDS gel, blotting the gel onto nitrocellulose paper using prestained molecular weight markers, incubating the paper with dilutions of LPS, washing, incubating the paper with purified hyper-immune rabbit IgG to the O-polysaccaride of the LPS, and then developing the paper with an insoluble marker coupled to goat anti-rabbit IgG. Using a synthesized LPS binding peptide mimicking Limulus anti-LPS factor as a positive control, the assay was both sensitive and specific. There was no non-specific binding using either no LPS or heterologous LPS or using either no primary IgG or IgG to a heterologous strain of LPS. Development of the band containing low microgram amounts of peptide was seen in seconds, and after 10 minutes of incubation we can detect nanogram quantities of peptide (and hopefully other LPS binding proteins in inflammatory sera). We are in the process of refining the technique and hope soon to try it with inflammatory sera. For these experiments, the sera will be run in non-denaturing conditions before blotting. An example of one of our blots is shown on page 7.

B. Interactions of LPS in polyclonal antiserum to E. coli J5

This antiserum protects against LPS from all gram negative strains in animal models and in clinical trials, and is the basis for the development of monoclonal antibodies to "core" epitopes on LPS. There are however no solid phase binding assays which confirm LPS-immunoglobulin binding, and existant monoclonal antibodies have not been documented to bind to LPS in a convincing manner. The ability of these Mabs to protect is controversial. We hope that a fundamental understanding of how LPS interacts with substances in this antisera will lead to insights as to how the antisera protect. This knowledge should provide us with methods to prepare truly protective antibody preparations.

We used the methods described in our initial grant application to study LPS-lipoprotein interactions in inflammatory sera to polyclonal antisera to E. coli J5. We found that much more $^{3}$H-LPS was precipitated from this antisera

with calcium and dextran than in normal sera. Further experiments showed that the precipitation depended absolutely on the presence of small amounts of normal serum in the reaction mixture, and that delipidation of the normal serum removed this facilitating function. IgG from J5 antisera, but not normal sera, had activity. This is the first description of a radioimmunoassay to measure the interactions of LPS with immunoglobulin in this antiserum and thus for the first time we have a definative technique to carefully analyze the binding. These results were published in the June 1991 issue of J. Infectious Disease.

At the end of the last trimester, we found that $^{3}$H-LPS can be immunoprecipitated from the solutions directly (in the presence of immune IgG and normal serum), so that calcium and dextran are not necessary to precipitate the complexes. We believe that this is an important advance because it means that we can now carefully study the immunoprecipitate without having to worry about all the non-specific proteins which would be precipitated by adding calcium and dextran. A goal that we set at the end of the last trimester was to study the range of LPS which were immunoprecipitated, and to compare the binding of IgG with IgM using the radioimmunoassay. This information is relevant to the potential future development of Mabs which would be protective for all strains.

Immunoprecipitation of our panel of different strains of $^{3}$H-LPS in antiserum to E. coli J5 is shown below. Results are given as the mean of two assays to the nearest whole percentage point. Every LPS in our panel is immunoprecipitated by this antiserum, indicating broad cross-reactivity.

| | % LPS immunoprecipitated from normal serum | % LPS immunoprecipitated from J5 antiserum |
|---|---|---|
| E. coli strains | | |
| O1 | 2 | 28 |
| O2 | 29 | 80 |
| O4 | 6 | 47 |
| O6 | 2 | 37 |
| O7 | 1 | 31 |
| O8 | 2 | 24 |
| O16 | 2 | 34 |
| O18 | 5 | 38 |
| O25 | 6 | 69 |
| O75 | 8 | 37 |
| O111 | 2 | 53 |
| O113 | 1 | 46 |
| S. typhimurium | 6 | 28 |
| K. pneumonia | 7 | 23 |

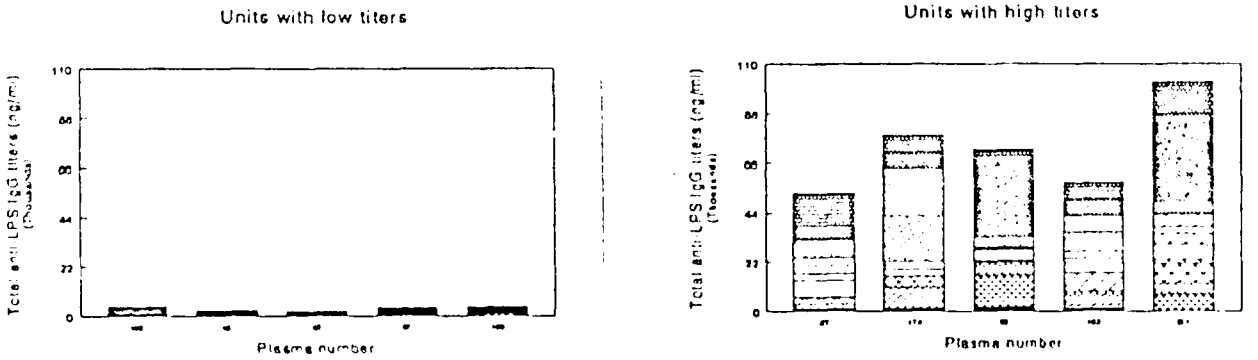
We purified the IgG from J5 antiserum using sepharose-protein A and have documented by SDS gel electrophoresis that it is > 98% pure. We are in the midst of the relatively difficult task or preparing highly purified IgM. We have delipidated it by ultracentrifugation in KBr x 2, salted it out in 35% $NH_3SO_4$, twice passed it over a 100 cm sephacryl molecular sizing column, and are passing it over the sepharose-protein A column to remove trace IgG. It is now about 95% pure, with a high molecular weight band and a trace band at about 40KD as contaminants. We plan as a final step to use cation exchange on the HPLC. We will then adjust the IgG and IgM concentrations to those in the parent antiserum and directly compare the role of each in the immunoprecipitation.

C. Analysis of IgG titers to different O-antigens present in screened human plasma

We found in a preliminary study in which we screened 202 normal human plasma units simultaneously by ELISA to multiple different O-antigens of LPS that the range of titers varied about 100-fold. The range and median titer of the screening to our panel of LPS is shown below.

| LPS | Range of IgG (ng/ml) | Median titer |
|---|---|---|
| *E. coli* strains | | |
| O1 | 128-20500 | 592 |
| O4 | 244-15300 | 1651 |
| O6 | 13-5746 | 348 |
| O12 | 43-7909 | 420 |
| O15 | 134-8213 | 995 |
| O16 | 154-38000 | 1995 |
| O18 | 66-20500 | 1170 |
| O25 | 55-13300 | 938 |
| O75 | 123-9029 | 684 |
| O111B4 | 44-2132 | 346 |
| S. minnesota | 133-1422 | 879 |
| S. typhimurium | 67-40000 | 484 |
| P. aerugenosa | 124-40000 | 1109 |

A small percentage of the units (about 5%) contained high titers to most of the O-antigens whereas other units contained low titers to most of the antigens. Analysis of this data is extensive and complex and will be given in the annual report. However, the difference between the five plasmas with the highest and lowest titers to the multiple LPS O-antigens selected by the screening was striking and is summarized in the figure below.



These results suggested that "natural" IgG titers to LPS vary substantially, and that it may be possible to creat a protective IgG preparation by screening of normal plasma donors which have high levels of natural antibody to multiple clinically relevant LPS. To test this hypothesis, it will be necessary to prepare pools of plasma selected for high and low IgG and test the the pools in physiological models. It will probably be necessary to eventually screen a larger number of plasma units. However, we have prepared two such pools and begun to test them in different models. At this point, we have performed a single assay using an actinomycin D sensitized mouse model. In this assay there was a minimal difference between the pools, but the dose-response was poor and the saline control was suspect. A problem with the assay was that the technician was learning how to inject the mice and therefore it will need to be repeated. Because of the potential importance of the findings, we are also planning to test the protective efficacy of the screened pools to diminish LPS induced fever, leukopenia, and hemodynamic changes in a sheep model (as outlined in the original grant proposal).

II. **New knowledge**

1. We have prepared a panel of radiolabeled LPS comprised of the 10 strains of E. coli which account for 60% of bacteremia and sepsis from E. coli. These reagents, which do not exist elsewhere, should be extremely helpful in analyzing the behavior of LPS in sera.

2. We have developed a Western blotting assay in order to study and help purify substances which bind LPS. The assay is highly specific and sensitive to nanogram quantities of protein (using a LPS binding peptide mimicking Limulus anti-LPS factor as a positive control. The assay will hopefully allow us to study LPS binding substances in different sera, and will almost certainly allow better study and/or development of other peptides which bind LPS. It is also conceivable that the technique could be adapted for use as a diagnostic assay for circulating LPS.

3. We previously had detected LPS-IgG binding in J5 antisera by precipitating complexes with calcium and dextran (conditions known to precipitate lipoproteins). We have now found that radiolabeled LPS from a wide variety of gram negative strains is immunoprecipitated without the addition of a precipitating agent when incubated in J5 antisera. A normal serum factor is an absolute requirement for the precipitation, suggesting that the LPS may undergo a physicochemical shift before binding to immunoglobulin. One possibility is that the LPS interacts with a LPS binding factor in normal serum, exposing an epitope which can then bind to immunoglobulin. This new finding strengthens our prior findings, and also should allow us to better analyze the precipitate for clues as to the facilitating factor present in normal serum (since there should not be irrelevant proteins precipitated.)

III. **Technical Problems**

Essentially none. Our initial actinomycin D sensitized murine assay comparing screened high and low titer plasma pools was difficult to interpret. I believe that this was because the technician doing the work had not previously done this assay. We plan to repeat it and I am confident that this is not a major difficulty. Otherwise, all is going very well.

IV. **Future Directions**

We are excited about our progress and findings on several fronts as noted above. The requirement for an additional factor present in serum for immunoglobulin in J5 antiserum to bind to LPS is intriguing. To our knowlege, the immunoassay we are using is the only method to demonstrate binding of LPS to immunoglobulin (or anything else) in this antiserum, which we believe is important because the antiserum is protective clinically. Future issues to be addressed are: 1. the importance of IgG vs IgM (previous dogma, without supporting data, has suggested that IgM has more activity than IgG), 2. what is the nature of the factor in serum which facilitates the IgG-LPS binding, and 3. how to best go about using this knowledge to develop hopefully protective anti-LPS Mabs.

The route to take concerning the finding that there are some random blood donors which have high levels of natural IgG to multiple O-antigens on LPS seems clear. A major issue is whether these differences, which seem striking, are physiologically important, and especially whether high-titered units of plasma are protective in models of endotoxicity. We are in the process of studying this in murine and sheep models.

V. **Goals for the next trimester**

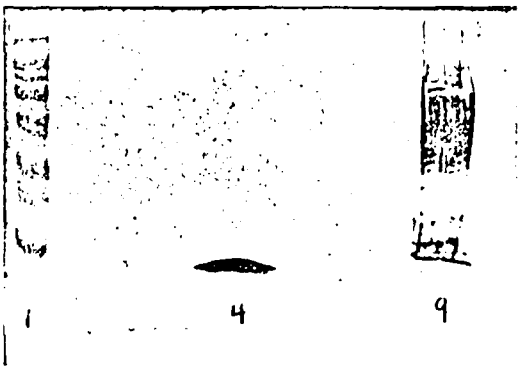
1. Further development of the Western blotting assay for detecting substances which bind LPS. Adaption of the method to non-denaturing gels, and initial attempt at detecting LPS binding substances in normal and inflammatory serum.

2. Finish purification of IgM from J5 antiserum. Prepare large standard lot of IgM and characterize with respect to purity, titer, and absence of IgG. Compare IgG and IgM side-by-side for potency with previously purified and standardized IgG from same antiserum.

3. Absorption experiments with different gram negative bacterial strains and immunoglobulin from J5 antiserum to define specificity of epitopes.

4. Characterization of precipitates formed by incubating $^{3}H$-LPS in J5 antiserum (using SDS gel electrophoresis.)

5. Protective efficacy of screened high titer plama against challenge with LPS in actinomycin D sensitized mice and in sheep model of fever, neutropenia, and hemodynamic changes




Sample Western blot to capture LPS from E. coli O111. SDS gel was run with prestained markers on left (lane 1), irrelevant synthetic peptides (15 ug, lanes 2,6,7), LPS binding peptide mimicking Limulus anti-LPS factor (15 ug, lane 4), and E. coli O111 LPS (1.75 ug, lane 9). Gel was blotted onto nitrocellulose paper, and incubated successively with E. Coli O111 LPS (10 ug/ml), rabbit anti-E. coli O111 LPS IgG, biotinylated goat anti-rabbit IgG, and avidin-peroxidase precipitating substrate. All 4 peptides run with the dye front. Only the LPS binding peptide in lane 4 captures LPS, and is visible as single dark band in the middle of the gel. LPS run in SDS in gel (lane 9) blots and develops as well in characteristic ladder pattern of wild type LPS due to repeating subunit structure of O-side chain. Substitution of heterologous LPS or primary IgG resulted in no staining. Subsequent blots suggest that we can detect 20 ng of peptide.